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TRANSGENIC PLANTS

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to a genetic mechanism for mitigating the effects of introgression of a genetically engineered genetic trait of a crop to a weed and of mitigating a weedy potential of the crop and, more particularly, to a genetic mechanism for mitigating the effects of introgression of genetically engineered resistancies of crops to weeds.

Crop domestication and weeds: During the prehistoric and historic processes of domestication of crops, farmers selected against a large number of traits that were valuable for wild species, but detrimental to agronomic practice. These differences between wild species and crops were further accentuated by selective breeding, and even more so by genetic engineering, which allowed introducing traits that were non-existent in the gene pool of the species, genus, family, or kingdom of the crop.

Concurrently with domestication; a few wild species evolved to fill the new ecological niches, the disturbed ecosystems known as farmers' fields (Baker, 1974; Holt, 1988; Turner, 1988). Only a few hundred of the tens of thousands of wild species have followed this evolutionary pathway from wild plant to widespread agricultural weed (Holm *et al.*, 1997). Thus, even though some weeds are closely related to crops or are even of the same species as the crops, they vary in a number of traits that distinguish them from wild species, as well as from the crop. These evolutionary processes are not static; indeed they are quite dynamic even on a human generation timescale (Baker, 1991). Changes in agricultural practices (drainage, fertilizer use, tillage and herbicide use) caused some pernicious weeds to return to being wild species, and some wild species to become weeds (Haas and Streibig, 1982). Crops can become "volunteer" weeds in the following crop, or even feral, and re-evolve some weedy traits. Some weeds have even introgressed new traits from conventionally-bred crops (wild barleys in barley have introgressed many new traits; wild sunflowers from sunflowers (Snow *et al.*, 1998). Worse, crops have introgressed weedy traits from related weeds e.g., poor oil quality in canola (Diepenbrock and Leon, 1988), and early bolting in sugar beets from weedy beets (Boudry *et al.*, 1998). These dynamic evolutionary events all occurred before the advent of transgenics.

Crops often possess conventionally-bred traits that would be advantageous to the weeds growing in their midst. Horizontal gene transfer

(introgression to totally unrelated species) occurs only rarely to other species within a genus, and even more rarely to species in closely related genera. Thus, even vital traits for weeds such as herbicide resistance have never passed horizontally from non transgenics (Torgersen, 1996), for example from wheat to grass weeds all in the family *Poaceae*. This lack of horizontal transfer allows the control of these related weeds in the crop. The weeds have had to evolve herbicide resistance from within their own genomes, and not by horizontal gene transfer.

The genetic distances between crop and weed were slightly enhanced with the advent of genetic engineering. Traits could be artificially forced horizontally into the crops to enhance cost-effectiveness of agriculture (higher yields, new products, resistances to insects, diseases, and to herbicides). Detractors of both the process of genetic engineering and its products have raised the possibilities that the engineered crops would become uncontrollable weeds, or that the genes would introgress into related weeds rendering them weedier, or into wild species, turning them into weeds (Kloppenburg, 1988; Goldberg *et al.*, 1990; Risler and Mellon, 1993). Hyper-generalizations were raised and terminology such as "superweeds" were coined (Kling, 1996). Calls were issued to prohibit or abandon all transgenic crops because of the possibilities of introgression of such traits into some weeds (Risler and Mellon, 1993). The fact that most crops have no interbreeding relatives in much of the world (Keeler *et al.*, 1996) did not allay fears for those crops. The issues made it to the popular press with kinky statements such as "The greatest danger of genetic engineering of plants may come from sex with weeds". The debate became as sterile as most of the interspecific hybrids generated using highly unnatural lab tricks to save the F_1 hybrids (Darmency, 1994).

Risk analysis and risk mitigation: Tomes were written on how to assess the risks of introgression – some with continuing generalizations and some discussing how and why this must be done case by case (Regal, 1994; Keeler *et al.*, 1996; Kareiva *et al.*, 1996; de Kathen, 1998; Williamson, 1993; Timmons *et al.*, 1996; Kjellsson *et al.*, 1998; Sindel, 1997; Gressel and Rotteveel, 1999, Galun and Breman, 1997; Krimsky and Wrubel, 1996). These discussion of the hazards and risk assessment do not discuss how biotechnologies can be used to mitigate the risk. No-one, including the governmental panels that permitted cultivating transgenic crops (Anonymous, 1994a, b;, 1997) or those interested in regulatory aspects (Be *et al.*, 1996; Waters, 1996) has seemed to ask if there are ways to prevent

weeds from using any traits that may introgress from crops, in the few instances where one can be quite sure that introgression eventually will occur.

As further detailed hereinunder, a case by case analysis of where intra or interspecific introgressions between genetically engineered crops and weeds are possible shows that there are specific genetic possibilities for mitigation of interspecific introgression.

There is thus a widely recognized need for, and it would be highly advantageous to have, failsafe mechanisms to reduce the possibility of intra and interspecific introgressions between genetically engineered crops and weeds.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a method of mitigating the effects of introgression of a genetically engineered genetic trait of a crop to a weed and of mitigating a weedy potential of the crop , the method comprising the step of producing apomictic seeds of the crop of a type which give rise to male sterile crop plants to thereby prevent introgression of the genetically engineered genetic trait of the crop to the weed.

According to another aspect of the present invention there is provided a method of mitigating the effects of introgression of a genetically engineered genetic trait of a crop having multiple genomes derived from different wild sources to a weed having a genome compatible with one of the multiple genomes, the method comprising the step of cytogenetically selecting for genetically engineered crop plants in which a gene or genes responsible for the genetic trait are localized on one or more of the multiple genomes of the crop which is not, or is far less, compatible with the genome of the weed.

According to still another aspect of the present invention there is provided a method of mitigating the effects of introgression of a genetically engineered genetic trait of a crop to a weed, the method comprising the step of cytogenetically selecting for or producing genetically engineered crop plants in which a gene or genes responsible for the genetic trait are genetically linked to an endogenous genetic trait of the crop, the endogenous genetic being deleterious to the weed.

According to yet another aspect of the present invention there is provided a method of mitigating the effects of introgression of a genetically

engineered genetic trait of a crop to a weed and of mitigating a weedy potential of the crop , the method comprising the step of controlling the expression of the genetically engineered genetic trait in the crop by at least one control element which is inexpressible by the weed.

According to still another aspect of the present invention there is provided a genetic construct for genetically modifying a crop to express a genetically engineered genetic trait while mitigating the effects of introgression of the genetically engineered genetic trait of the crop to a weed, the genetic construct comprising a first nucleic acid segment encoding for the genetic trait and at least one additional nucleic acid segment including at least one control element which is expressible by the crop, yet which is inexpressible by the weed.

According to an additional aspect of the present invention there is provided a method of mitigating the effects of introgression of a genetically engineered first genetic trait of a crop to a weed and of mitigating a weedy potential of the crop , the method comprising the step of co-engineering at least one copy of a genetically linked second genetic trait in the crop, the second genetic trait being innocuous or somewhat valuable to the crop yet deleterious to the weed.

According to a further aspect of the present invention there is provided a genetic construct for genetically modifying a crop to express a genetically engineered first genetic trait while mitigating the effects of introgression of the genetically engineered first genetic trait of the crop to a weed, the genetic construct comprising a first nucleic acid segment encoding for the first genetic trait and at least one additional nucleic acid segment encoding a second genetic trait, the second genetic trait being innocuous or somewhat valuable to the crop yet deleterious to the weed.

According to yet a further aspect of the present invention there is provided a crop genetically modified to include the above described genetic constructs and to express the traits encoded thereby.

According to further features in preferred embodiments of the invention described below, the second genetic trait is of abolished secondary dormancy.

According to still further features in the described preferred embodiments the second genetic trait is of uniform or delayed ripening.

According to still further features in the described preferred embodiments the second genetic trait is of anti-shattering of ripe seeds.

According to still further features in the described preferred embodiments the second genetic trait is of dwarfism.

According to still further features in the described preferred embodiments the second genetic trait is selected from the group consisting of seed stalk bolting, seed coat defects that facilitate uniform germination, root storage promotion, biennial growth and non-flowering.

The present invention successfully addresses the shortcomings of the presently known configurations by conceiving and providing a mechanism for mitigating the effects of introgression of a genetically engineered genetic trait of a crop to a weed and of mitigating a weedy potential of the crop .

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of a genetic mechanism which can be used for mitigating the effects of introgression of a genetically engineered genetic trait of a crop to a weed and of mitigating a weedy potential of the crop. Specifically, the present invention can be used to prevent introgression of genetically engineered resistancies of crops to weeds.

The principles and operation of the present invention may be better understood with reference to the accompanying descriptions and examples.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of the components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Generally, the nomenclature used herein and the laboratory procedures in recombinant DNA technology described below are those well known and commonly employed in the art. Standard techniques are used for cloning, DNA and RNA isolation, amplification and purification. Generally enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like are performed according to the manufacturers' specifications. These techniques and various other techniques are generally performed according to Sambrook *et al.*, molecular Cloning--A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989) which is incorporated herein by reference.

Other general references are provided throughout this document. The procedures and information therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

One of the greatest advantages of herbicide-resistant crops is that they allow control of closely-related weeds that have the same herbicide selectivity spectrum as the crop and could not be previously controlled. Similarly, and advantage of disease and insect resistant crops is that they can be grown where there are secondary hosts, often close relatives, harboring the pests. Other resistant crops, e.g., cold resistance crops, are also of great advantage. Similarly, highly productive crops are advantageous, as are crops with modified product such as different types of starch and oils. Such and other genetic traits have been introduced into crops of various types by transgenics. These advantages of transgenics are fine, if there is no introgression into a relative weed or if the crop itself does not become a "volunteer" weed in subsequent crops. Because the advantages of transgenics are so great in the above cases, both industry and the farmers are clamoring for the transgenics. They even do so when they know that introgression to weeds is imminent. However, if the gene for herbicide resistance introgresses, the farmers are just back to square one. Considering the strong competition of red-rice with rice, and the magnitude of yield loss (Pantone and Baker, 1991), the desire of the farmers can be understood. One cannot state that this argument is wrong; just that there are a limited number of herbicide resistances one can engineer and thus a limited number of times that one can return to square one.

Hence, while conceiving the present invention, the idea was realized to mitigate the risks of introgression of a genetically engineered trait of a crop to a weed by coupling the gene of choice having the desired trait in tandem constructs with "antiweediness" genes. This coupling can either be physical, where the two genes are covalently linked prior to transformation or by the juxtaposition effect commonly achieved by co-transformation. Both will heretofore be termed "tandem", as the result in tightly linked genes. These would render weedy recipients or volunteer weeds less fit to act as competitors with crops, weeds and wild species. As further detailed and exemplified hereinbelow, genes that prevent seed shatter, that prevent secondary dormancy, that dwarf the recipient and others would all be useful for that purpose, as they would often be beneficial or innocuous or somewhat valuable to the crop while detrimental to weeds, or to the crop

when it is a "volunteer" weed, i.e., when it becomes a weed in the following crop, or if it becomes feral.

As used herein in the specification and in the claims section that follows, the term "weed" includes undesirable plants growing wild, especially those growing on cultivated ground to the disadvantage of a crop, lawn, or flower bed. The term further includes various forms of the crop species that are undesirable to agriculture: feral forms that have escaped cultivation and have evolved weedy characters, other varieties of the crop that do not possess the same transgenes, and the transgenic crop when it is a volunteer weed in following crops.

Thus, according to one aspect of the present invention there is provided a method of mitigating the effects of introgression of a genetically engineered trait of a crop to a weed and of mitigating a weedy potential of the crop. The method is effected by producing apomictic seeds of the crop of a type which give rise to male sterile crop plants, to thereby prevent introgression of the genetically engineered trait of the crop to the weed.

Male sterility in plants implies an inability to produce or to release functional (fertile) pollen. Male sterility in plants results in failure of formation or development of functional stamens, microspores or gametes.

From a structural/functional point of view, male sterility in plants may be divided into three categories which include (i) pollen sterility, wherein functional pollen grains are missing; (ii) structural (or staminal) male sterility, wherein male flowers or stamens are malformed and therefor non-functional, or missing altogether; and (iii) functional male sterility, wherein good and viable pollen is trapped in indehiscent anthers and thus prevented from functioning.

From a genetic point of view, male sterility in plants may also be divided into three categories which include (i) nuclear male sterility (NMS), also known in the art as genic or Mendelian male sterility, wherein male sterility is governed solely by one or more nuclear genes; (ii) cytoplasmic male sterility (CMS), wherein male sterility results due to a combined action of nuclear and cytoplasmic organelle (e.g., mitochondria or chloroplasts) genes; and (iii) non-genetic male sterility which is either chemically or mechanically (pollen removal) induced.

As further detailed hereinunder male sterility genes has been isolated and characterized. Such genes can be used to produce the apomictic seeds according to the present invention using genetic engineering techniques

which are well known in the art, some of which are further described hereinunder.

According to another aspect of the present invention there is provided a method of mitigating the effects of introgression of a genetically engineered trait of a crop having multiple genomes derived from different wild sources to a weed having a genome compatible with one of the multiple genomes. The method is effected by cytogenetically selecting for genetically engineered crop plants in which a gene or genes responsible for the trait are localized on one or more of the multiple genomes of the crop which is not, or is far less compatible with the genome of the weed.

For example, the D genome of wheat is compatible with the D genome of *Aegilops cylindrica* (goatgrass) a problematic weed in the western plain states of the U.S. Transgenes can introgress from wheat to this *Aegilops* (Zemetra *et al.*, 1998). Likewise, transgenes easily introgress from the B genome of oilseed rape to many *Brassica* weeds and wild species (Darmency, 1994; Bing *et al.*, 1996; Brown and Brown, 1996; Jorgensen and Andersen, 1994; Kerlan *et al.*, 1993; Landbo *et al.*, 1996; Lefol *et al.*, 1996a, b Metz *et al.*, 1997; Mikkelsen *et al.*, 1997; Scheffler *et al.*, 1995). Selecting for wheat and oilseed rape transgenic plants in which no transgenes are integrated in the D or B genomes, will mitigate the possibility of introgression of the transgenic trait to *Aegilops cylindrica* and *Brassica* weeds and wild species, respectively.

Conventional methods of gene mapping in plants and the availability of genetic markers being specific to the chromosomes of the various genomes can be employed using well known and developed cytogenetic techniques to select for genetically engineered crop plants in which a gene or genes responsible for the trait are localized on one or more of the multiple genomes of the crop, which genome is not compatible with the genome of the weed.

According to yet another aspect of the present invention there is provided a method of mitigating the effects of introgression of a genetically engineered trait of a crop to a weed. The method according to this aspect of the invention is effected by cytogenetically selecting for or producing genetically engineered crop plants in which a gene or genes responsible for the trait are genetically linked to an endogenous trait of the crop, the endogenous being deleterious to the weed.

According to still another aspect of the present invention there is provided a method of mitigating the effects of introgression of a genetically

engineered trait of a crop to a weed and of mitigating a weedy potential of the crop . The method according to this aspect of the invention is effected by controlling the expression of the genetically engineered trait in the crop by at least one control element which is inexpressible by the weed.

Accordingly, the present invention provides a genetic construct for genetically modifying a crop to express a genetically engineered trait while mitigating the effects of introgression of the genetically engineered trait of the crop to a weed. The genetic construct includes a first nucleic acid segment encoding for the trait and at least one additional nucleic acid segment including at least one control element which is expressible by the crop, yet which is inexpressible by the weed. These constructs can be tandemly produced, or the same effect can be achieved by co-transformation and co-lucus integration.

One example of a control element which is expressible by crop, yet is inexpressible by weeds is the 35S promoter which was originally derived from cauliflower mosaic virus (CaMV) and which is silenced in plants infected by the virus (Al-Kaff *et al.*, 1998) - i.e., most cruciferous weed plants in the wild. One of the basic and fundamental mechanisms in the process of speciation (species formation) is loss or gain of genetic control functions. It is therefor expected that a plurality of genetic control element will be functional in one species, yet non-functional in a closely related species.

According to still another aspect of the present invention there is provided a method of mitigating the effects of introgression of a genetically engineered first genetic trait of a crop to a weed and of mitigating a weedy potential of the crop . The method according to this aspect of the present invention is effected by co-engineering at least one copy of a genetically linked second genetic trait in the crop, wherein the second genetic trait being innocuous or somewhat valuable to the crop yet deleterious to the weed.

As used herein the term genetically linked refers to a genetic distance lower than 50 centiMorgan, preferably lower than 40 centiMorgan, more preferably lower than 30 centiMorgan, more preferably lower than 20 centiMorgan, more preferably lower than 10 centiMorgan, more preferably lower than 5 centiMorgan, more preferably lower than 1 centiMorgan, most preferably in the range of 0 to 1 centiMorgan, wherein 0 centiMorgan refers to juxtaposed sequences.

Accordingly, the present invention also provides a genetic construct for genetically modifying a crop to express a genetically engineered first genetic trait while mitigating the effects of introgression of the genetically engineered first genetic trait of the crop to a weed. The genetic construct includes a first nucleic acid segment encoding for the first genetic trait and at least one additional nucleic acid segment encoding a second genetic trait, the second genetic trait being innocuous or somewhat valuable to the crop, yet deleterious to the weed. The nucleic acid segment encoding the first genetic trait is preferably flanked on both sides by nucleic acid segments encoding the second genetic trait, to thereby reduce the risk of loosing the second genetic trait due to mutation or crossing over.

However, it will be appreciated that in many cases while using conventional transformation techniques genetic traits carried on two different vectors end up integrating to the same locus.

Thus, according to a further aspect of the present invention there is provided a crop genetically modified to include the above described genetic constructs and to express the traits encoded thereby.

The second genetic trait is, as already pointed out, innocuous or somewhat valuable to the crop, yet deleterious to the weed. Numerous examples of such genetic traits are listed herein and are further discussed in the Examples section that follows.

One such trait is abolished secondary dormancy. Genetically abolished secondary dormancy would be neutral to the crop, or advantageous to some crops having some residual secondary dormancy, but deleterious to the weed. Tillage, crop rotation, and preplant use of herbicides, all standard practices, would control the uniformly-germinating weed seeds lacking secondary dormancy during the following season.

Another such trait is uniform or delayed ripening. For example, methods and constructs for controlling the ripening of fruits and vegetables are disclosed in U.S. Pat. No. 5,512,466 which teaches the expression of an ACC metabolizing enzyme in the fruit to inhibit the production of ethylene.

Yet another such trait is of anti-shattering of ripe seeds. Uniform ripening and anti-shattering genes would be a negative trait for weeds, neutral for uniformly ripening and non-shattering crops (e.g., rice), and positive, for example, for oilseed rape, which still has a shattering problem.

Still another such trait is dwarfism. Examples for dwarfism genes include genes relating to hormone production (Azpiroz *et al.*, 1998; Schaller *et al.*, 1998) as well as those dealing with shade avoidance, such as, but not

limited to, over expressed phytochrome genes which prevents recognition of shading and thus the plant remains short (Robson *et al.*, 1996). Additional examples are provided in the Examples section that follows.

Additional traits innocuous or somewhat valuable to the crop, yet deleterious to the weed include, but are not limited to, seed stalk bolting, seed coat defects that facilitate uniform germination, root storage promotion, biennial growth and non-flowering.

Thus, these anti-weediness traits are combined, according to the present invention, with the genetically engineered traits, which genetically engineered traits include, but are not limited to, traits imposing resistance to herbicides and plant pests and pathogens, traits imposing resistance to environmental conditions, such as, but not limited to, cold, salinity, etc., and traits affecting yield, ripening, as well as modified components such as starches and oils, etc. Examples of such traits for which genes has been isolated are summarized in many recent texts such as Galun and Breiman, 1997. Examples of such traits for which genes has yet not been isolated, however, their isolation is readily available, include shattering of seeds, precluding secondary dormancy and promoting biennial growth.

Once a gene responsible for a mitigating trait has been selected, it must be engineered for plant expression along with the trait which confer an advantage thereto. To introduce such genes into a plant, a suitable chimeric gene and transformation vector must be constructed. A typical chimeric gene for transformation into a plant will include a promoter region, a heterologous structural DNA coding sequences and a 3' non-translated polyadenylation site. A heterologous structural DNA coding sequence means a structural coding sequence that is not native to the plant being transformed. Heterologous with respect to the promoter means that the coding sequence does not exist in nature in the same gene with the promoter to which it is now attached. Chimeric means a novel non-naturally occurring gene which is comprised of parts of different genes. In preparing the transformation vector, the various DNA fragments may be manipulated as necessary to create the desired vector. This includes using linkers or adaptors as necessary to form suitable restriction sites or to eliminate unwanted restriction sites or other like manipulations which are known to those of ordinary skill in the art.

Promoters which are known or found to cause transcription of selected gene or genes in plant cells can be used to implement the present invention. Such promoters may be obtained from plants, plant pathogenic

bacteria or plant viruses and include, but are not necessarily limited to, the 35S and, 19S promoters of cauliflower mosaic virus (CaMV35S and CaMV19S), the full-length transcript promoter from the figwort mosaic virus (FMV35S) and promoters isolated from plant genes such as EPSP synthase, ssRUBISCO genes and promoters obtained from T-DNA genes of *Agrobacterium tumefaciens* such as nopaline and mannopine synthases. The particular promoter selected should be capable of causing sufficient expression to result in the production of an effective amount of the respective proteins to confer the traits.

Particularly useful promoters for use in some applications of the present invention are fruit specific promoters and the full-length transcript promoter from the figwort mosaic virus (FMV35S). The FMV35S promoter is particularly useful because of its ability to cause uniform and high levels of expression in plant tissues. The DNA sequence of a FMV35S promoter is presented in U.S. Pat. No. 5,512,466 and is identified as SEQ ID NO:17 therein. Examples of fruit specific promoters include the E8, E4, E17 and J49 promoters from tomato (Lincoln *et al.*, 1988), as well as the 2A11 promoter as described in U.S. Pat. No. 4,943,674.

The promoters used for expressing the genes according to the present invention may be further modified if desired to alter their expression characteristics. For example, the CaMV35S promoter may be ligated to the portion of the ssRUBISCO gene which represses the expression of ssRUBISCO in the absence of light, to create a promoter which is active in leaves but not in roots. The resulting chimeric promoter may be used as described herein. As used herein, the phrase "CaMV35S" or "FMV35S" promoter includes variations of these promoters, e.g., promoters derived by means of ligation with operator regions, random or controlled mutagenesis, addition or duplication of enhancer sequences, etc.

The 3' non-translated region contains a polyadenylation signal which functions in plants to cause the addition of polyadenylated nucleotides to the 3' end of an RNA sequence. Examples of suitable 3' regions are the 3' transcribed, non-translated regions containing the polyadenylation signal of the tumor-inducing (Ti) plasmid genes of *Agrobacterium*, such as the nopaline synthase (NOS) gene, and plant genes like the 7s soybean storage protein genes and the pea E9 small subunit of the RuBP carboxylase gene (ssRUBISCO).

The RNAs produced by a DNA construct of the present invention also preferably contains a 5' non-translated leader sequence. This sequence

can be derived from the promoters selected to express the genes, and can be specifically modified so as to increase translation of the mRNAs. The 5' non-translated regions can also be obtained from viral RNA's, from suitable eukaryotic genes, or from a synthetic gene sequence. The present invention is not limited to constructs wherein the non-translated region is derived from the 5' non-translated sequence that accompanies the promoter sequence. Rather, the non-translated leader sequences can be part of the 5' end of the non-translated region of the native coding sequence for the heterologous coding sequence, or part of the promoter sequence, or can be derived from an unrelated promoter or coding sequence as discussed above.

In a preferred embodiment according to the present invention, the vector that is used to introduce the encoded proteins into the host cells of the plant will comprise an appropriate selectable marker. In a more preferred embodiment according to the present invention the vector is a plant expression vector comprising both a selectable marker and an origin of replication. In another most preferred embodiment according to the present invention the vector will be a shuttle vector, which can propagate both in *E. coli* (wherein the construct comprises an appropriate selectable marker and origin of replication) and be compatible for propagation or integration in the genome of the plant organism of choice. In yet another embodiment, the construct comprising the promoter of choice, and the gene of interest is placed in a viral vector which is used to infect the cells. This virus may be integrated in the genome of the organism of choice or may remain non-integrated.

According to some embodiment of the present invention secretion of the protein or proteins out of the cell is preferred. In this embodiment the construct will comprise a signal sequence to effect secretion as is known in the art. For some applications, a signal sequence that is recognized in the active growth phase will be most preferred. As will be recognized by the skilled artisan, the appropriate signal sequence should be placed immediately downstream of the translational start site (ATG), and in frame with the coding sequence of the gene to be expressed.

Introduction of the construct into the cells is accomplished by any conventional method for transfection, infection or the like as is known in the art. In constructs comprising a selectable marker the cells may be selected for those bearing functional copies of the construct. If the plasmid comprising the gene of interest is episomal the appropriate selective conditions will be used during growth. Stable transfectants and stable cell

lines may be derived from the transfected cells in appropriate cases, in order to conveniently maintain the genotype of interest. Cell growth is accomplished in accordance with the cell type, using any standard growth conditions as may be suitable to support the growth of the specific cell line.

A DNA construct of the present invention can be inserted into the genome of a plant by any suitable method. Suitable plant transformation vectors include those derived from a Ti plasmid of Agrobacterium tumefaciens, such as those disclosed by U.S. Pat. No. 4,940,838 and others. In addition to plant transformation vectors derived from the Ti or root-inducing (Ri) plasmids of *Agrobacterium*, alternative methods can be used to insert the DNA construct of this invention into plant cells. Such methods may involve, for example, the use of liposomes, electroporation, chemicals that increase free DNA uptake, particle gun technology, and transformation using viruses. Methods for the introduction of vectors into maize, or other monocot cells would include, but are not limited to, injection methods or microprojectile methods.

The construction of vectors capable of being inserted into a plant genome via *Agrobacterium tumefaciens* mediated delivery is known to those of ordinary skill in the art. Typical plant cloning vectors comprise selectable and scoreable marker genes, T-DNA borders, cloning sites, appropriate bacterial genes to facilitate identification of transconjugates, broad host-range replication and mobilization functions and other elements as desired.

If Agrobacterium mediated delivery is chosen, once the vector has been introduced into the disarmed *Agrobacterium* strain, the desired plant can then be transformed. Any known method of transformation that will work with the desired plant can be utilized.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Mitigation via failsafes

There are various failsafe mechanisms that can be used to mitigate the risk of introgression of traits between genetically engineered crops and weeds. Most of the discussion will describe the introduction of specific mitigating transgenes, but other mechanisms can also be envisaged, as described below.

Apomixis as a failsafe: One area that may develop is apomixis (Koltunow *et al.*, 1995), where seed is actually of vegetative origin. This is being developed to establish hybrid vigour without crosses. If apomictic varieties are pollen free, then genes cannot introgress into other species or into varieties of a crop. The lack of viable pollen is probably the only failsafe that would be acceptable to some detractors, who fear intervarietal movement of transgenes.

Cytogenetic Failsafes: Some crops such as wheat and oilseed rape are composed of multiple genomes derived from different wild sources (Kimber and Sears, 1997; U, 1935). In any given locale it is possible that only one of the genomes of the crop is identical to that of a related weed allowing easy gene transfer. For example: the D genome of wheat is compatible with the D genome of *Aegilops cylindrica* (bearded goatgrass) a problematic weed in the western plain states of the U.S. Transgenes can introgress from wheat to this *Aegilops* (Zemetra *et al.*, 1998). Likewise, transgenes easily introgress from the B genome of oilseed rape to many *Brassica* weeds and wild species (Darmency, 1994; Bing *et al.*, 1996; Brown and Brown, 1996; Jorgensen and Andersen, 1994; Kerlan *et al.*, 1993; Landbo *et al.*, 1996; Lefol *et al.*, 1996a, b Metz *et al.*, 1997; Mikkelsen *et al.*, 1997; Scheffler *et al.*, 1995). The further the genetic distances of between crop and weed in these crosses, the greater the needs for techniques such as embryo rescue to save hybrids abortion, and the greater the incidence of infertile offspring. The natural integration of a transgene on the D genome of wheat or the B genome of oilseed rape in interspecific crosses is quite simple. This should not be the case if the transgene is on the incompatible A or B genomes of wheat or the C genome

of oilseed rape. When the transgene is on one of the incompatible genomes, rare homologous recombination (crossing over) is required to integrate the transgene into stable and fertile offspring. Thus, cytogenetic mapping of transgenes, and releasing only those transgenic lines where the transgene is on genome incompatible with local weeds will lower the risk of introgression with weeds by orders of magnitude (Gressel and Rotteveel, 1999). Surprisingly, such risk lowering has not appeared among the requirements of regulatory authorities (Anonymous, 1994a, b, 1997; Be *et al.*, 1996; Waters, 1996).

Transgenetic Mitigation (TM)

The concept of using genetic engineering to mitigate any positive effects transgenes may confer is based on three premises:

i. Tandem constructs of genes act genetically as tightly-linked genes and their segregation from each other is exceedingly rare.

ii. There are traits that are either neutral or positive for a crop that would be deleterious to a typical or volunteer weed, or to a wild species.

iii. Because weed have a strong competition amongst themselves, and have a large seed output of weeds, even mildly deleterious traits are quickly eliminated from populations.

Thus, if the gene of choice being engineered into a crop is flanked on either side by a TM gene in a tandem construct, the overall effect would be deleterious to weeds introgressing the construct from a crop. Even if one of the TM genes mutates, is deleted, or crosses over, the other flanking TM gene will remain, providing mitigation.

One TM trait has already been put into transgenic crops, albeit inadvertently: the use of the 35S promoter in gene constructs of desirous traits in oilseed rape (*Brassica napus)*. The 35S promoter was originally derived from cauliflower mosaic virus (CaMV). This promoter is silenced in plants infected by the virus (Al-Kaff *et al.*, 1998) - i.e., most cruciferous weed plants in the wild. Thus, a wild and weedy related *Brassica* that introgresses herbicide resistance from oilseed rape is suddenly herbicide sensitive in a CaMV-infected weed. One could consider the advertent use of such promoters.

Other TM traits that could be used are best visualized when observing the differences between crops and weeds. This is best illustrated with two cases: (i) rice and weedy red rice (both *Oryza sativa*) as well as

rice with wild rices *Oryza* spp.; (ii) oilseed rape (*Brassica napus)* and feral and weedy Polish rape/wild radish *B. campestris* = *B. rapa,* as summarized below.

Seed dormancy: Weed seeds typically have secondary dormancy with seeds from one harvest germinating bit by bit throughout the following season, and over a number of years (Vleeshouwers *et al.*, 1995). This evolutionary trait is considered to be a risk-spreading strategy that maximizes fitness while reducing losses due to sib competition (Hyatt and Evans, 1998; Lundberg *et al.*, 1996). Staggered secondary dormancy prevents all the weeds from being controlled by tillage before the crop is planted, or controlled by tillage or herbicides during crop rotation. Rare mutants lacking secondary dormancy were selectively propagated during crop domestication, as the loss of secondary dormancy is desirable to the farmer, who wants uniform germination after planting the crop. Crop seed that germinates uniformly after planting gives a uniform harvest. This can well be seen when comparing crops with their weedy progenitors and relatives (Ling-Hwa, 1997). Genetically abolishing secondary dormancy would be neutral to the crop, but deleterious to the weed. Tillage, crop rotation, and preplant use of herbicides, all standard practices would control the uniformly-germinating weed seeds lacking secondary dormancy during the following season.

Ripening and shattering: Weeds disperse their seed over a period of time and much of the ripe seed "shatters" to the ground. This ensures replenishment of the soil seed bank. A proportion of the weed seed is harvested with crop seed, contaminating crop seed, facilitating weed dispersal to wherever the crop seed will be grown. Weeds have evolved morphological and phenological "mimicries" to the crop seed (Barrett, 1983; Gould, 1991) necessitating continual evolution and refinement of seed cleaning techniques to remove the contaminating weed seed. Crop varieties have been selected for non-shattering, but recently domesticated crops such as oilseed rape still suffer from shattering (Simon, 1994; Prakash, 1988; Price *et al.*, 1996). The first problem in domestication is control of shattering (Young, 1991; Levy, 1985). In addition to the loss of yield, the shattering of crop seed causes the crop to be a volunteer weed in the following crop (Lutman, 1993).

Uniform ripening and anti-shattering genes would be a negative trait for the weeds, neutral for rice (because it ripens uniformly and does not shatter easily after thousands of years of selection), and positive for oilseed

rape, which still has a shattering problem. The addition of anti-shattering genes in a TM construct could also prevent cultivated oil seed rape from becoming a volunteer weed problem as well as being a TM gene. Crop seed contaminated with low levels of weed seed are typically used for feeding or processing, only weed free "certified" seed is sown.

Dwarfing:

Dwarfing has been especially valuable in generating "green revolution" crops, but also has value in normal cropping situations. The green revolution in rice and wheat is based on a modification of the harvest index, the ratio of grain to straw. For millenia these crops had been selected for height, to outgrow weeds, limiting the photosynthate available for grain. Weed evolution continued apace, giving rise to taller weeds. The advent of selective herbicides to kill weeds allowed for genetic dwarfing of these crops resulting in more seed harvest and less straw. Some of these dwarfing genes are tightly linked to genes reducing general yield potential. Still, the lowering of height, precluding the concomitant problem of tall plants "lodging" (falling over), and increased yield, especially after fertilizer use (which previously promoted lodging), allowed countries like India to become self sufficient, despite population increase.

Various new systems of genetically engineered height reduction are being introduced. These include genes relating to hormone production (Azpiroz *et al.*, 1998; Schaller *et al.*, 1998) as well as those dealing with shade avoidance. Much of stem elongation is in response to shading. This is advantageous when competing with other species, but not in a weed-free crop stand where only siblings are competing. The overexpression of specific phytochrome genes prevents recognition of shading and thus the plant remains short (Robson *et al.*, 1996). This is advantageous for a crop and could also be used where the present dwarfing genes prevent obtaining the highest yields. This trait would be disadvantageous for a weed that must compete with the crops; it would be shaded over by the crop.

QTLs: QTLs or other unidentified genes that have been shown to provide general weediness characters are known (Paterson *et al.*, 1995). QTLs have also been identified controlling dormancy (Van der Schaar *et al*, 1997) and for the late stages of gibberellin biosyntheses (Lange *et al.*, 1997), which might relate to stem dwarfing or seed stalk bolting.

Other TM traits: One could envision other traits that would be disadvantageous to weeds but neutral to crops. These include removing seed coat characters that allow weed seeds to pass through animal digestive

tracks intact and then be dispersed. Genes that promote root storage would be advantageous to cultivated beets but detrimental to annual wild beets. Genes that prevent bolting (i.e., promote biennial growth) would be excellent for carrots, celery, cabbage, lettuce, bee, and related crops, but would be highly deleterious to related weeds. Anti-flowering genes would prevent introgression of genes from potatoes into wild Andean relatives (the only place where hazards of introgression from potatoes exist to weeds). They would also prevent volunteer potatoes arising from true seed, where this is a problem. Sorghum provides an interesting case. Crosses between cultivated sorghum (*S. bicolor)* and johnsongrass (*S. halapense*) gave rise to sterile, perennial, vegetatively-propagating plants (Baker, 1991). The hybrids have thick rhizomes storing large amounts of material. Various of the QTL's reported by Peterson *et al* (1995) could severely decrease the fitness of any hybrids that form.

Presumably, comparisons of crop traits with weed traits will lead to finding other TM traits.

Balancing desirous transgenic traits with TM traits

There is considerable debate about the advantages that would accrue to weeds from the primary transgenic traits. Resistance by modified site of the herbicide binding site to its target should only confer an advantage when the herbicide is used. It is unknown whether there would be pleiotropic advantages of herbicide resistance due to introducing genes for metabolic inactivation of herbicides. Insect, or pathogen resistances would clearly provide an advantage to a weed, if the weed does not already have these resistances, and is affected by the pest. Many pest resistances were bred out of wild species during domestication; the chemicals weeds use to alleviate pest problems often taste bad or are toxic to mammals.

Still, let us assume that the primary transgenic trait confers an advantage to a weed; how much will TM traits actually mitigate that advantage? Weeds are not only highly competitive with crops, they are competitive with weeds of other species as well as within their own species. Seed-producing weeds often produce thousands of seeds, in steady state conditions, to replace a single plant, suggesting extreme competition to be the replacement; the selection for high competitive fitness is intense. This has dual implications in the discussed situation. A weed that introgresses any transgenic trait, will proliferate and spread though a population very quickly, even if it has fitness advantage that is marginally positive (Thill

and Mallory-Smith, 1997; Crawford *et al.*, 1997). Conversely, one can balance the disadvantage of TM traits against the advantage of the primary trait. This must be done in both in the presence and absence of the reason for using the primary trait. The primary trait only provides an advantage when it is needed; when there is pressure from the pest, herbicide, or stress. Some primary traits that were not meant to confer any plant protective advantage to the crop might still do so when introgressed into a weed, e.g., changed oil or starch composition. Membrane lipid compositions do change in response to temperature and other stresses, and the ability to adapt to certain environments might be enhanced or decreased (Cooper and Raybould, 1997; Linder, 1998); a plant with a modified starch might be less palatable to insects or less digestible by fungi, and thus have an advantage. In the absence of the factor making the primary trait desirable it should not have any advantage, accentuating the utility of the TM traits. Indeed, when the primary selector is not present, the primary transgenic trait can be disadvantageous; this has been demonstrated with one herbicide resistance gene (Bergelson *et al*, 1996). Many herbicides have a short residual effect and when they are not present, there is no advantage to having transgenic herbicide resistance. Similarly, while some insect pests disease pathogens are continually present, there are many others that appear in damaging levels only in certain seasons, or even only once every few years, when the climatic conditions are just right for their causing pandemics. If resistance to a disease or insect has only occasional value, and the resistance mechanism has a fitness penalty when not needed, the value of the primary gene is depleted, and TM genes will elicit a net decrease in fitness.

Likelihood of TM genes segregating from advantageous genes

Often, the expression of one gene of a tandem construct is lost in the primary transgenic plant, and sometimes the expression is lost after a few generations. The reasons for these losses are not always clear nor relevant for this discussion, as only stabilized progeny of transformants are released to agriculture. If all traits of a tandem construct are expressed after 4-5 generations of development, it is fair to consider it stable, i.e., as stable as any native, tightly-linked adjacent genes. The likelihood of the TM and desired trait segregating from each other is infinitesimally lower than for the TM trait being inactivated by a mutation. Thus, if the mutation frequency of inactivation of the TM gene is 10^{-6} to 10^{-7}, that would be the frequency of the loss of the TM trait as the frequency of crossing over is many orders

of magnitude lower. If the frequency of crossing over of tightly linked traits is an unacceptably high risk, then one can consider using two TM genes, flanking the primary gene on either side. The frequency of loss of two genes TM can be predicted as 10^{-12} to 10^{-14}. Each TM trait should work in a balance with the primary trait, and where the primary gene gives a strong advantage to a weed, it might be necessary to have more than one TM trait in a construct. The risk of losing a TM trait can be further decreased by combination with a cytogenetic failsafe, where it is available. If the tandem construct is located on a non-homologous chromosome (where such exist), then only rare homologous recombination can move it. As there is no selective advantage to losing the TM trait on the non-homologous chromosome, one can compound the frequency of likelihood of homologous recombination with the frequency of loss of the TM trait(s).

Do TM genes have to give 100% safety: No risk situations are impossible but how low a risk do we need to attain? This is really a question for regulators, but when they do deliberate the issue they must ask; when will the related weed evolve the trait in question by natural means. This is best illustrated by an example: one company in the United states engineered resistance of ALS (acetolactate synthase)–inhibiting herbicides into sunflowers (*Helianthus annuus)* but never even field tested them, because of fear that the gene might introgress into weedy wild sunflowers (also *H. annuus*), prevalent in the mid western plain states. Natural introgressions between this crop and related weed are common and cause problems (Arias and Riesenberg, 1994; Whitton *et al.*, 1995). The mutations conferring resistance to ALS-inhibiting herbicides are naturally prevalent in plant populations in a frequency of one in a million, and ALS-inhibiting herbicides are widely used. Wild sunflowers have recently evolved resistance to ALS-inhibiting herbicides in monoculture cropping (White *et al.*, 1998). If a TM construct had been inserted in tandem with an ALS gene, with a likelihood of segregating of 10^{-10}, then the likelihood of getting ALS-resistant wild sunflowers would not have appreciably been changed by introgression from transgenics. Wild sunflowers could have been controlled in sunflower fields, lessening the possibilities of natural introgressions. Such analyses should be made wherever possible.

TM traits which are available as gene sequences

Some of the traits suggested for use as TM genes are just known to exist as named traits that are inherited, others are also mapped as genes to

positions on various chromosomes, and a few are actually characterized as sequenced genes. Thus, not all TM traits are immediately available for insertion in tandem constructs. Still, there can be many different ways for a plant to confer a TM trait, and thus, more than one gene might be available.

Many of the TM traits be introgressed into the crops are not yet available as genes for transformation, suggesting that efforts be instituted to isolate TM genes. Gene hunting to a large extent is no longer driven by a desire for basic knowledge; it is heavily driven by a perceived utility of the gene. Now that the TM genes have been given a value, they might be rapidly sought and found, especially with the availability of transposon tagging as a method for hunting and fishing.

An interim solution may be to select transformants that have randomly introgressed the primary gene to a point in close linkage to a TM trait. Many of these TM traits are already mapped in major crops, such as dormancy in rice (Wan *et al*, 1998; Lin *et al.*, 1998). The closer the linkage distance, the lesser the likelihood of segregation. In the future, when technologies are available for site specific insertion of genes into chromosomes, the primary genes could be spliced close to TM genes, without having to know the sequence of the TM gene.

Secondary dormancy: While the genetics of secondary dormancy has been described, it is not clear which genes actually control it (Li and Foley, 1997; Khan, 1998). In important weeds such as *Avena fatum*, the lack of dormancy is dominant (Foley and Fennimore, 1998) such that this, as a transgene on the domesticated oats (*A. sativa*) would turn wild oats into a less fit weed. Unfortunately, *Arabidopsis,* the typical source for genes, has already been sufficiently domesticated that it is unlike cruciferous weeds; the lab strains no longer have strong secondary dormancy (Van der Schaar *et al.*, 1997). An *Arabidopsis* mutant that is insensitive to abscisic acid, lacks secondary dormancy (Steber *et al.*, 1998). Perhaps a way to find more genes is to use the genetic differences between wild *Arabidopsis* strains and the lab strains presently used, as is being done in other instances (Auckerman *et al.*, 1997).

An endo-β-mannanase has been shown to be part of wall softening during seed germination (Bewley, 1997). Such a gene could overcome secondary dormancy in weeds, while accelerating the rate of primary germination in crops, itself a valuable trait.

Much has been published on the physiology of secondary dormancy and how its causes vary among species. In some cases it is due to

impervious seed coats and in others due to various inhibitors found in the seed.

Shattering: Physiologically, one way to avoid seed shattering is to have uniform ripening. Early maturing soybean and oilseed rape seeds on indeterminate continuously flowering varieties typically shatter. Thus, determinacy, with its single uniform flush of flowering is one method to prevent shattering, but this often shortens the season, reducing yield. The hormonology of the abscission zone controls whether shattering will occur and it is possible that if cytokinins are overproduced, then shattering will be delayed. In sorghum the genes that control shattering seem to be on QTLs (Paterson *et al.*, 1995) as discussed above.

Stature limitation:

Vertical deprivation (dwarfing) has proven itself as a desirable trait in crops due to the increase in harvest index by virtue of having less stem and more seed. The genes used so far seem to have an unknown function. Still many genes are known, via their physiological role, that could control height.

Gibberellins: It is well known that preventing the biosyntheses of gibberellins reduces the height. The genetic relationships between some dwarfing genes and gibberellin biosyntheses has been elucidated (Webb *et al.*, 1998). This is the basis of many chemical dwarfing agents used commercially to lower stature and prevent lodging of wheat. The enzymes and genes controlling various steps in gibberellin biosyntheses are also known. Copalyl diphosphate synthase, ent-kaurene synthase, and ent-kaurene oxidase are responsible for early stages in the biosynthesis of all gibberellins (Smith *et al.*, 1998; Yamaguchi *et al.*, 1998, Hedden and Kamiya, 1997; Lange, 1998; Helliwell *et al.*, 1998). *Arabidopsis* mutations bearing mutations in any of them are dwarfed, with the dwarfing being reversible by gibberellin treatment. Overexpression of a gene coding for ent-kaurene synthase, causing co-suppression mimicked the mutant phenotype.

Some processes, such as flower stalk bolting, are controlled by specific gibberellins; in radish, GA_1 and GA_4 are responsible for flower stalk bolting (Nishijima *et al.*, 1998). It may be necessary to characterize the genes coding for the monooxygenases and dioxygenases that are responsible for these later steps (Hedden, 1997). Some of these genes have been isolated as well (Kusaba *et al.*, 1998).

Brassinosteroids: This new group of hormones also causes elongation of stems in many plant species, and their absence results in dwarf plants. A 22 α-hydroxylase cytochrome P450 has recently been isolated that controls a series of these steps in brassinosteroid biosynthesis (Choe *et al.*, 1998), and plants missing the enzyme are dwarfed (Azpiroz *et al.*, 1998). Additionally, suppressive overexpression of a sterol C24-methyl transferase also causes dwarfing (Schaller *et al.*, 1998).

Shade avoidance:

Various forms of the pigment phytochrome interact to detect whether a plant is being shaded (Smith and Whitelam, 1997; Devlin *et al.*, 1998; Torii *et al.*, 1998; Auckerman *et al.*, 1997). Phytochrome recognition of shading leads to stem elongation, which is unneeded in a weed-free crop. The engineering of suppressive overexpression constructs of one of these phytochromes led to plants that did not elongate as a results of shading (Robson *et al.*, 1996). Much of the gene isolation has been from *Arabidopsis*, yet the suppressive overexpression was active in dwarfing tobacco.

TMs for vegetatively propagated crops: The genes for pollen sterility (Williams, 1995) would clearly be the simplest way to render potato to be without true seeds. Such potatoes could not become volunteer weeds (from true seeds), or have pollen that introgresses into other potato varieties or into wild related Andean species (Love, 1994; Eijlander and Steikema, 1994).

TMs for biennial crops: Biennial crops such as beets, carrots, etc. usually require a period of cold vernalization before flower stalk formation (bolting). At the end of the vernalization there is typically a burst of endogenous gibberellin biosynthesis, which induces stalk elongation, and exogenous gibberellins can often replace the cold requirement. Possibly, bolting could be suppressed by including a TM antisense or suppressive overexpression construct for one of the enzymes of gibberellin biosynthesis, both on crop and of related weed. These genes are known (see above).

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications cited herein are incorporated by reference in their

entirety. Citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

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